

**Table S1. Selection of published Affimers, demonstrating range of applications and targets.**

Affimer target	Main use	Structures	Imaging	Kd*	Reference
Herceptin	Diagnostics	-	-	12 – 27 nM	(Adamson et al., 2019a)
Therapeutic antibodies	Diagnostics	-	-	-	(Adamson et al., 2019b)
Myoglobin & GFAP glial fibrillary acidic protein	Diagnostics	-	-	pM to nM	(Al-Enezi et al., 2021)
CEA (carcinoembryonic antigen)	Diagnostics	-	IF	6 – 34 nM	(Shamsuddin et al., 2021)
Cow Pea Mosaic Virus	Diagnostics	6QOZ	-		(Hesketh et al., 2019)
Clumping factor A (CfA) virulence factor	Diagnostics		IF	62 nM	(Caudwell et al., 2022)
Glypican-3	Diagnostics	-	-	-	(Xie et al., 2017)
Crimean-Congo hemorrhagic fever orthonaïrovirus nucleoprotein	Diagnostics, Inhibitor	6ZO0	-	5.7 nM	(Alvarez-Rodriguez et al., 2020)
p300	Protein-protein interaction	5A0O	-	89 - 140 nM	(Kyle et al., 2015)
EPAC1 (Exchange protein directly activated by cAMP 1)	Isoform selectivity	-	IF	-	(Buist et al., 2021)
KRas (GDP and GTP analogue forms)	Isoform selectivity, Inhibitor	7NY8,6YXW,6YR8	-		(Haza et al., 2021)
BCL-2 family proteins	Isoform selectivity	6ST2,6HJL,6STJ	-	90 – 3400 nM	(Miles et al., 2021)
FcyRIIIa	Inhibitor	5ML9, 5MN2	-	217 nM -2.6 μM	(Robinson et al., 2018)
SUMO-1, SUMO-2/3	Isoform selectivity, Inhibitor	5ELJ,5EQL,5ELU	(in cell expression)	35 – 414 nM, and >10 μM	(Hughes et al., 2017)
K6- and K33-linked diUbiquitin	High affinity interactors	5OHM, 5OHV, 5OHL	IF	~20 pM- 1.6 nM	(Michel et al., 2017)
Cholera Toxin B	Protein delivery	-	IF	97-1130 nM	(Balmforth et al., 2021)
ACTN2 (α-actinin-2)	Imaging	6WST	IF, super-res	-	(Curd et al., 2021)
Actin Affimer and Polaris probe (from Actin Affimer)	Imaging	7C03	IF, super-res, live cell imaging, FP	300 nM to >10 μM	(Lopata et al., 2018) (Sugizaki et al., 2021)
Fibrinogen	Modulation	-	(in vitro)	38 – 52 nM	(Kearney et al., 2019)
Transient Receptor Potential Vanilloid 1 (TRPV1) (peptide screen)	Modulation	-	IF	-	(Tiede et al., 2017)
Src2 homology (SH2) domains (p85α, Grb2,7,10,14)	Isoform selectivity	-	-	-	
Tenascin C	In vivo imaging/Diagnostics	-	IHC, in vivo	5.7 nM	
Herpes virus of turkeys (HVT UL49)	Diagnostics	-	IF	1.5 – 7.5 nM	
Tubulin	Imaging (super-resolution)	-	IF, super-resolution	-	
Human epidermal growth factor receptor 4 (HER4)	Imaging (super-resolution)	-	IF, super-resolution	-	
2,4,6- trinitrotoluene (TNT)	Small organic compound detection	-	-	-	

\*Kd typically measured by SPR or ITC (except for the Actin Affimer – measured by so-sedimentation with F-actin). IF, immunofluorescence; IHC, immunohistochemistry; FP, fluorescence polarisation. PDB entries for co-crystals (or CryoEM) structures, where available are included.

**Table S2. Selection of published nanobodies, demonstrating range of applications and targets**

Nanobody target	Main use	Structures	Fluorescence Imaging	Kd*	Reference
C4b (complement) (Immunisation)	Inhibitor	5JTW	-	3.2 nM - 14 pM	(De la O Becerra KI, 2022)
Na <sub>v</sub> 1.4 and Na <sub>v</sub> 1.5 ion channels (Immunisation)	Isoform capture from lysates	7R63	-	40 nM - 60 nM	(Srinivasan et al., 2022)
RHOA NaLi-H1	RhoA-GTP biosensor	-	chromobody	28 nM – 68 nM	(Keller et al., 2019)
BC2-tag ( $\beta$ -catenin) (Immunisation)	Imaging	-	3D dSTORM, chromobody	1.9 nM - ~3.1 nM	(Virant et al., 2018) (Traenkle et al., 2015)
ALFA-tag (Immunisation)	Imaging	6I2G	STED, DNA Paint, IF, chromobody	0.026 nM - 11 nM	(Götzke et al., 2019)
EGFR (Immunisation)	Therapeutics	4KRN, 7OM5, 7OM4	In vivo imaging	<60 nM, 166–276 nM	Reviewed in (Sharifi et al., 2021)
$\alpha$ -synuclein (Immunisation)	Diagnostic	-	IHC	1.9 $\mu$ M - 240 nM (fibril affinity higher)	(Hmila et al., 2022)
HER2 (Immunisation)	Imaging/Diagnostic	-	IF	1.9 nM – 14 nM	(Kijanka et al., 2013)
Actin (Immunisation & NaLi-H1)	Imaging	-	IF, dSTORM (binds G-actin)	-	(Rocchetti et al., 2014) (Moutel et al., 2016)
GFP (Immunisation & NaLi-H1)	Imaging	3OGO, 3K1K	Chromobody, dSTORM, IF	0.23 nM	(Kubala et al., 2010) (Kirchhofer et al., 2010) (Rothbauer et al., 2006) (Moutel et al., 2016)
Tubulin (Immunisation, & NaLi-H1)	Imaging	-	dSTORM, IF	-	(Kesarwani et al., 2020) (Mikhaylova et al., 2015) (Moutel et al., 2016)
Nup proteins (immunisation)	Imaging	6X07 and others	dSTORM, IF, chromobodies	14 pM – 230 nM	(Pleiner et al., 2015) (Nordeen et al., 2020)
$\beta$ 2 adrenoceptor	Structure determination	3POG and others	Fluorescent intrabody Localization Microscopy	1 nM – 100 nM	(Rasmussen et al., 2011) (Gormal et al., 2020)

\*Kd typically measured by SPR or ITC. IF, immunofluorescence; IHC, immunohistochemistry. Example PDB entries for co-crystals (or CryoEM) structures, where available are included. NaLi-H1: humanized synthetic single domain antibody library. Remaining nanobodies obtained after initial immunisation.

## References

- Adamson, H., Ajayi, M. O., Campbell, E., Brachi, E., Tiede, C., Tang, A. A., Adams, T. L., Ford, R., Davidson, A., Johnson, M. et al.** (2019a). Affimer-Enzyme-Inhibitor Switch Sensor for Rapid Wash-free Assays of Multimeric Proteins. *ACS Sens* **4**, 3014-3022.
- Adamson, H., Nicholl, A., Tiede, C., Tang, A. A., Davidson, A., Curd, H., Wignall, A., Ford, R., Nuttall, J., McPherson, M. J. et al.** (2019b). Affimers as anti-idiotypic affinity reagents for pharmacokinetic analysis of biotherapeutics. *Biotechniques* **67**, 261-269.
- Al-Enezi, E., Vakurov, A., Eades, A., Ding, M., Jose, G., Saha, S. and Millner, P.** (2021). Affimer-Based Europium Chelates Allow Sensitive Optical Biosensing in a Range of Human Disease Biomarkers. *Sensors (Basel)* **21**.
- Alvarez-Rodriguez, B., Tiede, C., Hoste, A. C. R., Surtees, R. A., Trinh, C. H., Slack, G. S., Chamberlain, J., Hewson, R., Fresco, A., Sastre, P. et al.** (2020). Characterization and applications of a Crimean-Congo hemorrhagic fever virus nucleoprotein-specific Affimer: Inhibitory effects in viral replication and development of colorimetric diagnostic tests. *PLoS Negl Trop Dis* **14**, e0008364.
- Balmforth, M. R., Haigh, J., Kumar, V., Dai, W., Tiede, C., Tomlinson, D. C., Deuchars, J., Webb, M. E. and Turnbull, W. B.** (2021). Piggybacking on the Cholera Toxin: Identification of a CTB-Binding Protein as an Approach for Targeted Delivery of Proteins to Motor Neurons. *Bioconjug Chem* **32**, 2205-2212.
- Buist, H. K., Luchowska-Stanska, U., van Barten, B., Valli, J., Smith, B. O., Baillie, G. S., Rickman, C., Ricketts, B., Davidson, A., Hannam, R. et al.** (2021). Identification and Characterization of an Affimer Affinity Reagent for the Detection of the cAMP Sensor, EPAC1. *Cells* **10**.
- Caudwell, J. A., Tinkler, J. M., Johnson, B. R. G., McDowall, K. J., Alsulaimani, F., Tiede, C., Tomlinson, D. C., Freear, S., Turnbull, W. B., Evans, S. D. et al.** (2022). Protein-conjugated microbubbles for the selective targeting of *S. aureus* biofilms. *Biofilm* **4**, 100074.
- Curd, A. P., Leng, J., Hughes, R. E., Cleasby, A. J., Rogers, B., Trinh, C. H., Baird, M. A., Takagi, Y., Tiede, C., Sieben, C. et al.** (2021). Nanoscale Pattern Extraction from Relative Positions of Sparse 3D Localizations. *Nano Letters* **21**, 1213-1220.
- De la O Becerra Kl, O. W., van den Bos RM, Xenaki KT, Lorent JH, Ruyken M, Schouten A, Rooijakkers SHM, van Bergen En Henegouwen PMP, Gros P.** (2022). Multifaceted Activities of Seven Nanobodies against Complement C4b. *J Immunol* **15**, ji2100647.
- Gormal, R. S., Padmanabhan, P., Kasula, R., Bademosi, A. T., Coakley, S., Giacomotto, J., Blum, A., Joensuu, M., Wallis, T. P., Lo, H. P. et al.** (2020). Modular transient nanoclustering of activated beta2-adrenergic receptors revealed by single-molecule tracking of conformation-specific nanobodies. *Proc Natl Acad Sci U S A* **117**, 30476-30487.
- Götzke, H., Kilisch, M., Martínez-Carranza, M., Sograte-Idrissi, S., Rajavel, A., Schlichthaerle, T., Engels, N., Jungmann, R., Stenmark, P., Opazo, F. et al.** (2019). The ALFA-tag is a highly versatile tool for nanobody-based bioscience applications. *Nature Communications* **10**, 4403.
- Haza, K. Z., Martin, H. L., Rao, A., Turner, A. L., Saunders, S. E., Petersen, B., Tiede, C., Tipping, K., Tang, A. A., Ajayi, M. et al.** (2021). RAS-inhibiting biologics identify and probe druggable pockets including an SII-alpha3 allosteric site. *Nat Commun* **12**, 4045.
- Hesketh, E. L., Tiede, C., Adamson, H., Adams, T. L., Byrne, M. J., Meshcheriakova, Y., Kruse, I., McPherson, M. J., Lomonossoff, G. P., Tomlinson, D. C. et al.** (2019). Affimer reagents as tools in diagnosing plant virus diseases. *Sci Rep* **9**, 7524.
- Hmila, I., Vaikath, N. N., Majbour, N. K., Erskine, D., Sudhakaran, I. P., Gupta, V., Ghanem, S. S., Islam, Z., Emara, M. M., Abdesselem, H. B. et al.** (2022). Novel engineered nanobodies specific for N-terminal region of alpha-synuclein recognize Lewy-body pathology and inhibit in-vitro seeded aggregation and toxicity. *FEBS J.*
- Hughes, D. J., Tiede, C., Penswick, N., Tang, A. A., Trinh, C. H., Mandal, U., Zajac, K. Z., Gaule, T., Howell, G., Edwards, T. A. et al.** (2017). Generation of specific inhibitors of SUMO-1- and SUMO-2/3-mediated protein-protein interactions using Affimer (Adhiron) technology. *Sci Signal* **10**.
- Kearney, K. J., Pechlivani, N., King, R., Tiede, C., Phoenix, F., Cheah, R., Macrae, F. L., Simmons, K. J., Manfield, I. W., Smith, K. A. et al.** (2019). Affimer proteins as a tool to modulate fibrinolysis, stabilize the blood clot, and reduce bleeding complications. *Blood* **133**, 1233-1244.
- Keller, L., Bery, N., Tardy, C., Ligat, L., Favre, G., Rabbits, T. H. and Olichon, A.** (2019). Selection and Characterization of a Nanobody Biosensor of GTP-Bound RHO Activities. *Antibodies (Basel)* **8**.
- Kesarwani, S., Lama, P., Chandra, A., Reddy, P. P., Jijumon, A. S., Bodakuntla, S., Rao, B. M., Janke, C., Das, R. and Sirajuddin, M.** (2020). Genetically encoded live-cell sensor for tyrosinated microtubules. *J Cell Biol* **219**.

- Kijanka, M., Warnders, F. J., El Khattabi, M., Lub-de Hooge, M., van Dam, G. M., Ntziachristos, V., de Vries, L., Oliveira, S. and van Bergen En Henegouwen, P. M.** (2013). Rapid optical imaging of human breast tumour xenografts using anti-HER2 VHJs site-directly conjugated to IRDye 800CW for image-guided surgery. *Eur J Nucl Med Mol Imaging* **40**, 1718-29.
- Kirchhofer, A., Helma, J., Schmidthals, K., Frauer, C., Cui, S., Karcher, A., Pellis, M., Muyldermans, S., Casas-Delucchi, C. S., Cardoso, M. C. et al.** (2010). Modulation of protein properties in living cells using nanobodies. *Nat Struct Mol Biol* **17**, 133-8.
- Kubala, M. H., Kovtun, O., Alexandrov, K. and Collins, B. M.** (2010). Structural and thermodynamic analysis of the GFP:GFP-nanobody complex. *Protein Sci* **19**, 2389-401.
- Kyle, H. F., Wickson, K. F., Stott, J., Burslem, G. M., Breeze, A. L., Tiede, C., Tomlinson, D. C., Warriner, S. L., Nelson, A., Wilson, A. J. et al.** (2015). Exploration of the HIF-1alpha/p300 interface using peptide and Adhiron phage display technologies. *Mol Biosyst* **11**, 2738-49.
- Lopata, A., Hughes, R., Tiede, C., Heissler, S. M., Sellers, J. R., Knight, P. J., Tomlinson, D. and Peckham, M.** (2018). Affimer proteins for F-actin: novel affinity reagents that label F-actin in live and fixed cells. *Sci Rep* **8**, 6572.
- Michel, M. A., Swatek, K. N., Hospenthal, M. K. and Komander, D.** (2017). Ubiquitin Linkage-Specific Affimers Reveal Insights into K6-Linked Ubiquitin Signaling. *Mol Cell* **68**, 233-246 e5.
- Mikhaylova, M., Cloin, B. M., Finan, K., van den Berg, R., Teeuw, J., Kijanka, M. M., Sokolowski, M., Katrukha, E. A., Maidorn, M., Opazo, F. et al.** (2015). Resolving bundled microtubules using anti-tubulin nanobodies. *Nat Commun* **6**, 7933.
- Miles, J. A., Hobor, F., Trinh, C. H., Taylor, J., Tiede, C., Rowell, P. R., Jackson, B. R., Nadat, F. A., Ramsahye, P., Kyle, H. F. et al.** (2021). Selective Affimers Recognise the BCL-2 Family Proteins BCL-xL and MCL-1 through Noncanonical Structural Motifs\*. *Chembiochem* **22**, 232-240.
- Moutel, S., Bery, N., Bernard, V., Keller, L., Lemesre, E., de Marco, A., Ligat, L., Rain, J. C., Favre, G., Olichon, A. et al.** (2016). NaLi-H1: A universal synthetic library of humanized nanobodies providing highly functional antibodies and intrabodies. *Elife* **5**.
- Nordeen, S. A., Andersen, K. R., Knockenhauer, K. E., Ingram, J. R., Ploegh, H. L. and Schwartz, T. U.** (2020). A nanobody suite for yeast scaffold nucleoporins provides details of the nuclear pore complex structure. *Nat Commun* **11**, 6179.
- Pleiner, T., Bates, M., Trakhanov, S., Lee, C. T., Schliep, J. E., Chug, H., Bohning, M., Stark, H., Urlaub, H. and Gorlich, D.** (2015). Nanobodies: site-specific labeling for super-resolution imaging, rapid epitope-mapping and native protein complex isolation. *Elife* **4**, e11349.
- Rasmussen, S. G., Choi, H. J., Fung, J. J., Pardon, E., Casarosa, P., Chae, P. S., Devree, B. T., Rosenbaum, D. M., Thian, F. S., Kobilka, T. S. et al.** (2011). Structure of a nanobody-stabilized active state of the beta(2) adrenoceptor. *Nature* **469**, 175-80.
- Robinson, J. I., Baxter, E. W., Owen, R. L., Thomsen, M., Tomlinson, D. C., Waterhouse, M. P., Win, S. J., Nettleship, J. E., Tiede, C., Foster, R. J. et al.** (2018). Affimer proteins inhibit immune complex binding to Fc $\gamma$ RIIIa with high specificity through competitive and allosteric modes of action. *Proc Natl Acad Sci U S A* **115**, E72-E81.
- Rocchetti, A., Hawes, C. and Kriechbaumer, V.** (2014). Fluorescent labelling of the actin cytoskeleton in plants using a camelid antibody. *Plant Methods* **10**, 12.
- Rothbauer, U., Zolghadr, K., Tillib, S., Nowak, D., Schermelleh, L., Gahl, A., Backmann, N., Conrath, K., Muyldermans, S., Cardoso, M. C. et al.** (2006). Targeting and tracing antigens in live cells with fluorescent nanobodies. *Nat Methods* **3**, 887-9.
- Shamsuddin, S. H., Jayne, D. G., Tomlinson, D. C., McPherson, M. J. and Millner, P. A.** (2021). Selection and characterisation of Affimers specific for CEA recognition. *Sci Rep* **11**, 744.
- Sharifi, J., Khirehgesh, M**      **Safari, F. and Akbari, B.** (2021). EGFR and anti-EGFR nanobodies: review and update. *J Drug Target* **29**, 387-402.
- Srinivasan, L., Alzogaray, V., Selvakumar, D., Nathan, S., Yoder, J**      **Wright, K. M., Klinke, S., Nwafor, J. N., Labanda, M. S., Goldbaum A. et al.** (2022). Development of high-affinity nanobodies specific for NaV1.4 and NaV1.5 voltage-gated sodium channel isoforms. *J Biol Chem* **298**, 101763.
- Sugizaki, A., Sato, K., Chiba, K., Saito, K., Kawagishi, M., Tomabechi, Y., Mehta, S. B., Ishii, H., Sakai, N., Shirouzu, M. et al.** (2021). POLArIS, a versatile probe for molecular orientation, revealed actin filaments associated with microtubule asters in early embryos. *Proc Natl Acad Sci U S A* **118**.
- Tiede, C., Bedford, R., Heseltine, S. J., Smith, G., Wijetunga, I., RosB-R., AlQallaf, D., Roberts, A. P., Balls, A., Curd, A. et al.** (2017). Affimer $\text{F}$  proteins are versatile and renewable affinity reagents. *Elife* **6**.
- Traenkle, B., Emele, F., Anton, R., Poetz, O., Haeussler, R. S., Maier, J., Kaiser, P. D., Scholz, A. M., Nueske, S., Buchfellner, A. et al.** (2015). Monitoring interactions and dynamics of endogenous beta-catenin with intracellular nanobodies in living cells. *Mol Cell Proteomics* **14**, 707-23.
- Virant, D., Traenkle, B., Maier, J., Kaiser, P. D., Bodenhofer, M., Schmees, C., Vojnovic, I., Pisak-Lukáts, B., Endesfelder, U. and Rothbauer, U.** (2018). A peptide tag-specific nanobody enables high-quality labeling for dSTORM imaging. *Nature Communications* **9**, 930.
- Xie, C., Tiede, C., Zhang, X., Wang, C., Li, Z., Xu, X., McPherson, M. J., Tomlinson, D. C. and Xu, W.** (2017). Development of an Affimer-antibody combined immunological diagnosis kit for glycan-3. *Sci Rep* **7**, 9608.